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The complexity and structural diversity of ant venom peptidomes is revealed by mass spectrometry profiling

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RATIONALE: Compared with other animal venoms, ant venoms remain little explored. Ants have evolved complex venoms to rapidly immobilize arthropod prey and to protect their colonies from predators and pathogens. Many ants have retained peptide-rich venoms that are similar to those of other arthropod groups.
METHODS: With the goal of conducting a broad and comprehensive survey of ant venom peptide diversity, we investigated the peptide composition of venoms from 82 stinging ant species from nine subfamilies using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOFMS). We also conducted an in-depth investigation of eight venoms using reversed-phase high-performance liquid chromatography (RP-HPLC) separation coupled with offline MALDI-TOFMS.
RESULTS: Our results reveal that the peptide compositions of ant venom peptidomes from both poneroid and formicoid ant clades comprise hundreds of small peptides (<4 kDa), while large peptides (>4 kDa) are also present in the venom of formicoids. Chemical reduction revealed the presence of disulfide-linked peptides in most ant subfamilies, including peptides structured by one, two or three disulfide bonds as well as dimeric peptides reticulated by three disulfide bonds.
CONCLUSIONS: The biochemical complexity of ant venoms, associated with an enormous ecological and taxonomic diversity, suggests that stinging ant venoms constitute a promising source of bioactive molecules that could be exploited in the search for novel drug and biopesticide leads. Copyright © 2015 John Wiley & Sons, Ltd.

Animal venoms are sophisticated biochemical weapons that are currently under intense investigation for their potential development into novel sources or lead compounds for therapeutic agents and insect-selective biopesticides. The molecular diversity of venoms, particularly peptide toxins, is linked to a formidable array of molecular targets and pharmacological properties. Several drugs and a biopesticide have already been developed based on the high selectivity and potency of venom peptides and an in-depth exploration of animal venoms will undoubtedly lead to further discoveries.[1–3]

Venoms exhibit an extraordinary biochemical complexity ranging from small molecules to large proteins that have been fine-tuned by nature for greater efficacy and target selectivity. In most animal venoms, peptides are the predominant class of toxins and have been investigated intensively in snakes,[4] scorpions,[5] cone snails,[6,7] and spiders.[8–10] However, as the venomous animal biodiversity encompasses *ca* 173 000 species, the vast majority of animal venoms remain unexplored in spite of their potential. Several large venomous animal groups have been little studied including centipedes,[11] ticks,[12] sea anemones,[13] wasps,[14] and ants. This is largely due to the small size and difficulty of collecting venoms or glands from these invertebrates. However, cutting-edge technologies such as transcriptomic and proteomic approaches now offer the possibility of exploring these venoms in detail, with samples of a limited size. Many species are also cryptic, difficult to access or can only be identified by specialized taxonomists. In addition, they may not be seen as a potential health threat to humans and therefore have attracted less attention than snakes, scorpions, spiders and cone snails that have been responsible for many human fatalities.

Ants (Hymenoptera: Formicidae) are a good example of such a neglected group. Ants are extremely diverse and ubiquitous in terrestrial environments[15] and can be considered one of the most abundant groups of venomous animals on Earth. Ants are known to use venoms for both offensive and defensive purposes and are also among the leading predators of other invertebrates in most ecosystems.[16] Some ant subfamilies lack stingers and spray secretions containing formic acid from their venom glands (Formicinae) or deposit small chemicals from their pygidial glands (Dolichoderinae) onto their targets. However, *ca* 70% of all ant species (*ca* 9000 species) are capable of stinging, and inject their venoms *via* an abdominal apparatus including a stinger connected to a

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venom gland.[17] Despite this, it is fascinating to note that, although the total number of stinging ant species is actually higher than the combined number of snake, scorpion and cone snail species, very little is known about their venom composition. Apart from those individuals with an allergy to ant stings, this is largely due to their innocuity and their small size. So far, most work on ant venoms has concentrated on small organic molecules such as hydrocarbons and alkaloids.[18–20] However, ant venoms also contain a variety of peptides and proteins[21,22] and therefore are potentially as complex as venoms from other groups of arthropods. This variability in the composition of ant venoms may be further enhanced by differing selection pressures due to their enormous ecological diversity.[22,23]

Recent studies have revealed that stinging ant venoms are rich in peptides, similar to other venomous animals.[21] However, very few ant venom peptides have been characterised and only 72 peptide sequences have been reported to date. These are mainly linear peptides lacking disulfide bonds that have antimicrobial, cytolytic and insecticidal activities. Examples include poneratoxin,[24,25] ponericins,[26] bicarinalins[27] and dinoponeratoxins.[28,29] The venoms of other arthropods as well as mollusks and snakes are known to contain disulfide-rich peptides as their main constituents.[9,30–33] Although ant peptides have not yet been broadly studied, some preliminary data indicate that ant venoms may also contain various types of peptides cross-linked by disulfide bridges. Disulfide bonds constrain the peptide backbone into rigid three-dimensional scaffolds which endow the peptides with a better chemical stability, increase resistance to *in vivo* proteolytic degradation, and form tightly defined pharmacophores that can be finely modulated by single amino acid mutations.[34] These characteristics make cysteine-rich venom peptides the major active components of animal venoms that result in their pharmacological activity.[9] In particular, the inhibitor cystine knot (ICK) structural motif is relatively common in small cysteine-rich peptide toxins from a variety of animal venoms and plants.[35–39] While large numbers of ICK peptide toxins have been reported in other arthropods such as cone snails, sea anemones, spiders and scorpions, only two such ant venom peptides with a disulfide connectivity consistent with ICK toxins have been described: a *Dinoponera* ICK-like peptide and SKTXs.[40,41] Indeed, all the disulfide-rich peptides previously isolated from ant venoms were homo- and heterodimeric complexes such as the myrmexins, pilosulins, and ectatomins found in venoms from the subfamilies Myrmeciinae,[42,43] Pseudomyrmecinae[44] and Ectatomminae[45] (for a complete review, see Aili *et al*.[46]).

In light of the enormous chemical, taxonomical and ecological diversity of ants, we therefore hypothesized that ant venoms probably represent a promising source of unique peptides with original scaffolds and novel pharmacologies. The study of ant venoms therefore opens up a new, and largely unexplored, field in toxinology that may hold great potential in the search for novel drug leads and genetically engineered biopesticides.

In earlier studies, we reported a novel method for the investigation of venom peptides and have successfully applied it to the chemotaxonomic study of selected ant species.[21] The present study is part of a broader investigation of ant venoms, with the aim of conducting a wide survey of ant venom peptidomes among nine different stinging ant subfamilies. The study was designed to provide the comprehensive coverage of ant venom peptide composition, particularly focusing on the discovery of disulfide-linked peptides in these venoms. The MS profiling of these ant venoms has revealed the occurrence of hundreds of unknown small linear peptides as well as many novel peptides cross-linked by one, two or three disulfide bonds, suggesting the great depth of structural and probable pharmacological diversity in ant venoms.

EXPERIMENTAL

Ant collection and taxonomy

Venoms from 82 ant species were investigated in the present study, covering 31 genera from 9 of the 16 stinging ant subfamilies. Field collections of live worker ants were conducted in various areas of French Guiana, with additional samples taken from continental France as well as Trinidad and Tobago. In order to include the largest possible biodiversity and phylogenetic range, additional venoms were purchased from a commercial supplier (Southwest Venoms, Tucson, AZ, USA). The samples included in the present study represent *ca* 1% of the total stinging ant species currently described and *ca* 13% of all stinging ant genera (Table 1 and Fig. 1(A)). Other subfamilies not included in the study are either rare or not present in the collection areas accessible to our group (South America, Australia and France), such as African ants. Some subfamilies such as the Amblyoponinae and Cerapachyinae are difficult to investigate because they are subterranean, which makes collection work more prone to serendipity. In addition, the subfamily Myrmicinae is very large and diversified with *ca* 6500 species[17] and therefore it is difficult to obtain a representative coverage of this subfamily. Phylogenetic analyses were conducted according to the most recent phylogeny of ants which describes 21 subfamilies and divides ant subfamilies into three clades: leptanilloid, poneroid and formicoid.[16,47] Ants belonging to the leptanilloid clade were not included in this study. The complete list of the ant species investigated in the present study is provided in Supplementary Tables S1 and S2 (see Supporting Information).

Collected ants were stored at −20°C prior to the dissection of the venom glands and specimens from each of the 82 species were also stored in 96% ethanol for later morphological identification. Three to thirty venom glands from worker ants were dissected for each species, pooled and stored in 10% acetonitrile (ACN)/water (v/v) for whole venom analysis. For further exploration by liquid chromatography coupled to mass spectrometry (LC/MS), 8–50 venom glands per species were dissected and pooled. Samples were centrifuged for 5 min at 14 400 rpm; the supernatant was collected and then freeze-dried prior to storage at −20°C. Freeze-dried venoms of *Myrmecia* sp., *Diacamma* sp., *Dinoponera grandis*, *Streblognathus aethiopicus*, *Tetraponera* sp. and *Pogonomyrmex maricopa* were purchased from Southwest Venoms.

Rapid Communications in Mass Spectrometry

Table 1. Genera and extant species of stinging ants

Clade	Subfamily	No. of extant genera	No. of genera studied	% of genera studied	No. of extant species	No. of species studied	% of species studied
poneroid	Amblyoponinae	13	1		121	1	
	Paraponerinae	1	1		1	1	
	Ponerinae	28	10		1157	39	
	Martialinae	1	0		1	0	
	Proceratinae	3	0		135	0	
	Agroecomyrmecinae	2	0		3	0	
formicoid	Cerapachyinae	7	1		267	2	
	Ecitoninae	5	4		151	8	
	Myrmeciinae	2	1		92	4	
	Pseudomyrmecinae	3	2		229	4	
	Ectatomminae	4	2		270	10	
	Myrmicinae	142	9		6443	13	
	Leptanilloidinae	3	0		15	0	
	Aenictinae	1	0		174	0	
	Heteroponerinae	3	0		24	0	
leptanilloid	Leptanillinae	6	0		59	0	
	Total	**224**	**31**	**13.84**	**9142**	**82**	**0.90**

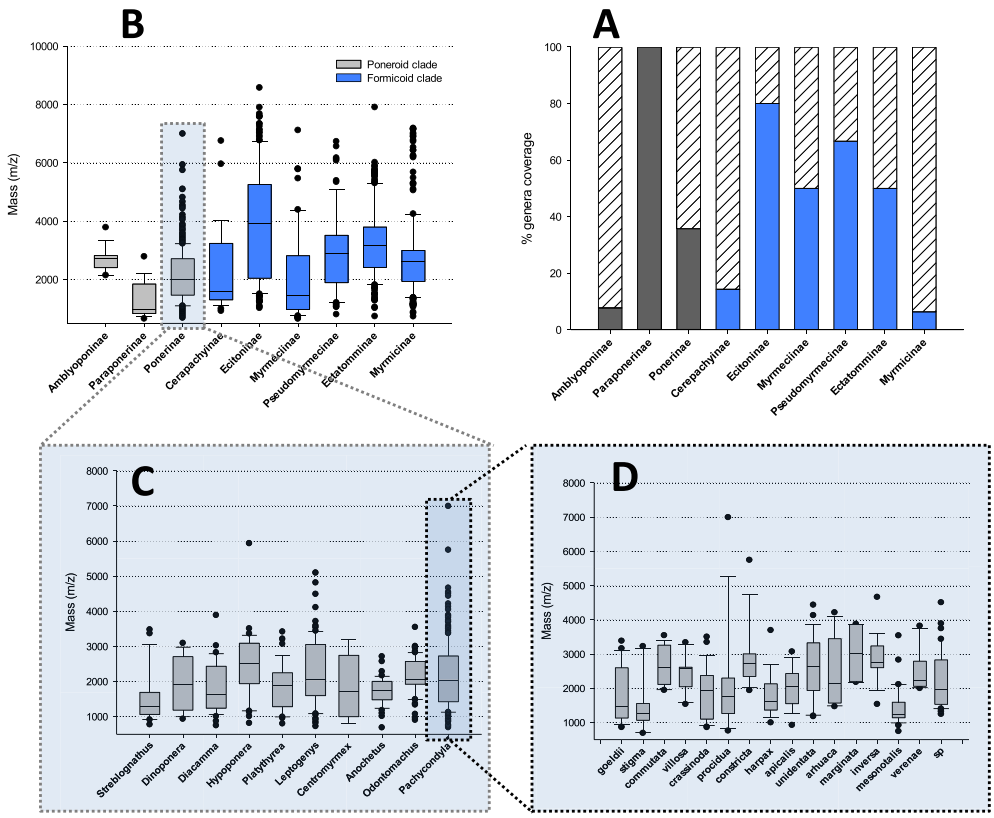

Figure 1. MALDI-TOFMS analysis of 82 crude venoms belonging to 9 stinging ant subfamilies from poneroid and formicoid clades. (A) Diversity coverage (%) of the nine subfamilies of stinging ants studied from poneroid (filled grey bars) and formicoid clades (filled blue bars). The white striped bars represent the percentage of genera not studied within each subfamily. (B) Box-and-whisker plot of the peptide mass distribution of all 82 ant venoms organised by subfamilies. The bottom and top end of each box represent the first and third quartiles, respectively, while the line inside each box represents the median mass. The ends of the whiskers represent the 5–95 percentile range while the black circles represent masses outside the 5–95 percentile range. (C) Box-and-whisker plot of the peptide mass distribution of ant venom peptides in the subfamily Ponerinae. (D) Box-and-whisker plot of the peptide mass distribution of ant venom peptides from 16 species of the genus *Pachycondyla*.

Mass spectrometry analysis of crude venoms

MS analyses were performed on a Voyager DE-Pro MALDI-TOF (matrix-assisted laser desorption ionisation time-of-flight) mass spectrometer (Applied Biosystems, Inc., Foster City, CA, USA). Samples were prepared as previously reported using a ferulic acid (FA) matrix dissolved in 20% ACN/water (v/v) containing 0.1% v/v trifluoroacetic acid (TFA) at a concentration of 10 mg/mL with the addition of 100 mM serine.[21] The FA/serine combination has previously been shown to be the most efficient matrix for the analysis of crude ponerine ant venoms by MALDI-TOFMS.[21] Prior to MS analysis, crude venoms were desalted using ZipTip® C18 pipette tips (Millipore, Billerica, MA USA). Then 0.5 µL of the desalted sample was deposited on the MALDI target plate followed by 0.5 µL of matrix. Each mass spectrum was calibrated externally using a mixture of peptides of known mass values in the same m/z range (Peptide Calibration Mix 4; LaserBio Labs, Sophia-Antipolis, France). Then 0.5 µL of the calibration mixture was co-crystallised with 0.5 µL of the matrix, spotted adjacent to each sample and measured separately. All calibration spectra were acquired in the automated mode, to maximise mass accuracy and reproducibility, and were calibrated automatically. Spectra of crude venoms were acquired in linear or reflector mode and calibrated automatically using the sequence module of the Voyager® Control software (Applied Biosystems, Foster City, CA USA). Five spectra of 50 laser shots per spectrum were accumulated for each sample based on the acceptance parameters and adequate signal intensity in the m/z 500–10 000 range. Mass spectra were collected in positive ion mode with 20 kHz acceleration. Signals below m/z 500 were not recorded as they were mostly matrix-related ion clusters.[8] All the ions observed were singly charged $[M+H]^+$ ions, for peptides in the mass range observed. No doubly charged ions were observed under the ionisation conditions used.

RP-HPLC separation

In order to obtain a representative picture of ant venom diversity, one venom was selected from each of the eight different ant subfamilies to be further analysed by LC/MALDI-MS. The C18 reversed-phase high-performance liquid chromatography (RP-HPLC) separation of venoms of the following species was therefore conducted: *Prionopelta* cf. *amabilis* (Amblyoponinae), *Pachycondyla goeldii* (Ponerinae), *Acanthostichus* sp. 1 (Cerapachyinae), *Eciton burchelli* (Ecitoninae), *Myrmecia pilosula* (Myrmeciinae), *Tetraponera* sp. (Pseudomyrmecinae), *Gnamptogenys sulcata* (Ectatomminae) and *Manica rubida* (Myrmicinae). These selected ant venoms were fractionated using an Xterra-C18 column (5 µm, 2.1 × 100 mm; Waters, Milford, MA, USA) with a gradient of solvent A (0.1% v/v TFA) and solvent B (ACN/0.1% v/v TFA). The percentage of solvent B was modified as follows: 0% for 5 min, 0–60% over 60 min, 60–90% over 10 min and 90–0% over 15 min at a flow rate of 0.3 mL/min. The eluate was monitored by UV absorbance at 215 nm on a diode-array detector. All analyses were performed on an HP 1100 HPLC system (Agilent, Santa Clara, CA, USA). Peptide elution was monitored in real time and fractions were collected manually for each eluting peak. Each fraction was then dried and reconstituted in 50 µL of 0.1% v/v TFA for offline MALDI-TOFMS analysis and disulfide bond reduction.

Disulfide bond reduction and alkylation

The presence of disulfide-bonded peptides in ant venoms was determined through the chemical reduction of crude venoms and HPLC fractions. 5 µL of crude venom or selected HPLC fractions were incubated with 10 µL of 100 mM ammonium bicarbonate buffer (pH 8) containing 6 M guanidine and 10 mM dithiothreitol (DTT) for 30 min at 56°C. The reaction was stopped by the addition of 5 µL of 0.1% v/v TFA. Prior to MS analysis, reduced venoms or fractions were desalted using ZipTip® C18 pipette tips (Millipore, Billerica, MA, USA). Chemical reduction results in a mass increase of 2 Da for each disulfide bond present in the molecule. Thus, by comparing the mass spectra of native and reduced samples, the number of disulfide bonds in ant venom peptides can be determined. However, due to the use of a single-stage MALDI-TOFMS instrument, the resolution of the instrument did not permit the detection of +2 Da mass differences in peptides with masses above 5 kDa. Therefore, the venom fractions which contained peptide masses over 5 kDa were also alkylated, following DTT reduction, by incubating the mixture with 50 mM iodoacetic acid (IAA) for 15 min at room temperature in the dark. The formation of the *S*-carboxymethyl derivative of cysteine results in a mass increase of 116 Da for each disulfide bond (58 Da for each cysteine residue) that allows the determination of the number of disulfide bonds in larger venom peptides.

Mass spectra analysis

All mass spectra were processed with Data Explorer® 4.11 software (AB SCIEX, Framingham, MA, USA) and subjected to a baseline correction with a correlation factor of 0.7 and Gaussian smoothing to reduce noise with a 5-point filter width. Supplementary $[M+Na]^+$ and $[M+K]^+$ adduct ions were manually removed from all mass lists. Values within ±1.0 m/z units of neighbouring HPLC fractions were considered as identical peptides, reflecting incomplete separation, and were also removed. Two-dimensional scatter plots, termed '2D venom landscapes', were constructed using SigmaPlot 12.0 software (Systat, San Jose, CA, USA). All peptide masses detected in each HPLC fraction were plotted as a function of their m/z values (x-axis) and their HPLC retention time reflecting their hydrophobicity (y-axis). Averaged data represents the mean ± S.D. unless otherwise stated.

RESULTS

Crude venom analysis

The crude venoms of the 82 ant species were initially analyzed by MALDI-TOFMS and a total of 1396 distinct masses were detected in all venoms after the elimination of masses consistent with doubly charged ions, or potassium and sodium adducts. The mean number of peptides detected in crude venom profiles was 17 ± 9 (mean ± standard

Rapid Communications in Mass Spectrometry

deviation (SD)) and varied widely, ranging from 4 to 42 in *Chalepoxenus muellerianus* (Myrmicinae) and *Solenopsis saevissima* (Myrmicinae) venoms, respectively.

The analysis of crude venoms revealed a great heterogeneity in the peptide molecular weight between subfamilies with an overall range of $[M+H]^+$ ions of *m/z* 652.2–8569.4 The huge variation in peptide composition between subfamilies is illustrated by the narrow *m/z* range of Amblyoponinae (*m/z* 2138.8–3782.6) as opposed to the broad *m/z* range observed in both Ponerinae and Ecitoninae subfamilies (*m/z* 685.7–6992.0 and *m/z* 1019.5–8569.4, respectively). The analysis of all the crude venoms revealed the presence of mostly low-mass ions with 87% of all ions below *m/z* 4000 (Fig. 1(B)). However, higher mass ions with *m/z* values >4000 were also observed in specific venoms, notably in subfamilies belonging to the formicoid clade, where 22.9 ± 23.7% (n = 41 species investigated) of the ions had *m/z* values >4000. The proportion of large peptides in ant venoms is even greater in Ecitoninae venoms with 56.3 ± 24.3% (n = 8 species investigated) of the $[M+H]^+$ ions in the range *m/z* 4000.8–8569.4. This finding is consistent with previous reports of large peptides described and sequenced from formicoid ant venoms such as ectatomins,[48] myrmexins[44] and pilosulins.[42,43,49] It should be noted that the higher peptide masses are associated with dimeric forms of peptides, often associating two linear chains linked by interchain disulfide bonds.[46] In the range of species studied, the proportion of large peptides ($[M+H]^+$ ions > *m/z* 4000) found in ant venoms from the poneroid clade was significantly lower than in the formicoid clade (Mann–Whitney U = 280, $n_1 = n_2 = 41$, $P < 0.001$). Only 3.0 ± 6.1% (n = 39 species investigated) of the peptides of ponerine ants were found to have $[M+H]^+$ ions > *m/z* 4000 (*m/z* range 4049.8–6992.0). This result confirms our earlier observations of Ponerinae ant venom composition, particularly from the *Pachycondyla* and *Odontomachus* genera, where 99% of all the molecular masses detected were in the mass range 0.5–5.0 kDa.[21] However, despite the narrow range of masses, there are significant differences between genera and between species belonging to the same genus as illustrated by results from ten different genera in the subfamily Ponerinae (Fig. 1(C)) and species from the large genus *Pachycondyla* (Fig. 1(D)).

Overall, the examination of crude ant venom peptidomes indicates that ant venoms may contain primarily small peptides in the mass range 0.5–4.0 kDa with larger peptides observed mostly in the formicoid clade. We can therefore estimate that the lengths of most ant venom peptides are below 35 residues. This estimate was determined from a MW_{av} of 111.1254 Da using the statistical occurrences of amino acids in the proteins of an average amino acid, averagine, calculated with the formula $C_{4.9384}H_{7.7583}N_{1.3577}O_{1.4773}S_{0.0417}$.[50] This is similar to conotoxins that are typically between 10 and 30 amino acids in length[51] but is in contrast to spider, scorpion and snake venom peptide toxins that are normally between 40 and 100 amino acids in length.[52]

Disulfide bond mapping in crude venoms

To broadly map the presence of disulfide-bonded peptides in crude venoms, the venoms were reduced. The comparative MALDI-TOFMS profiling of native venoms revealed the presence of *ca* 50 peptides linked by disulfide bonds. We detected disulfide-bonded peptides among five distinct ant subfamilies from both the formicoid and poneroid clades (i.e. Ponerinae, Amblyoponinae, Ectatomminae, Myrmeciinae and Myrmicinae). A comparison of the spectra before and after reduction permitted us to detect the presence of peptides with one, two or three disulfide bonds (Figs. 2(A)–2(C)). These masses are listed in Supplementary Table S3 (Supporting Information).

Only 32 peptides with one disulfide bond were detected in 14 crude venoms (i.e. *Pachycondyla commutata*, *P. mesonotalis*, *P. unidentata*, *Odontomachus hastatus*, *O. scalptus*, *Anochetus horridus*, *A.* cf. *diegensis*, *Streblognathus aethiopicus*, *Prionopelta* cf. *amabilis*, *Ectatomma brunneum*, *Myrmecia rufinodis*, *M. similima*, *Pogonomyrmex maricopa*, and *Manica rubida*); 17 peptides with two disulfide bonds were detected only in venoms from the genus *Anochetus* (i.e. *A. horridus*, *A.* cf. *diegensis* and *A. emarginatus*); and one peptide with three disulfide bonds was detected in *P. unidentata* venom. From our sample survey, more disulfide-linked peptides were detected in venoms from the poneroid clade (42 disulfide-bonded peptides) than in the formicoid clade (8 disulfide-bonded peptides). Overall, 33 disulfide-bonded peptides were found in Ponerinae venoms plus nine in Amblyoponinae venoms (all poneroids), whereas only one disulfide-bonded peptide was found in Ectatomminae, two in Myrmeciinae and five in Myrmicinae venoms (from the formicoid clade). This may indicate a greater structural diversity in the Ponerinae, although a larger venom sample set encompassing more species is needed for any definitive conclusion to be drawn.

While disulfide-bonded peptides seem to be minor components in most ant venoms, some venoms appeared to deviate from this pattern and were composed mostly of disulfide-bonded peptides. Of the 37 peptides detected in the venoms of the three *Anochetus* species (Ponerinae) 54% had one and two disulfide bonds and 90% of the 10 peptides detected in the venom of *Prionopelta* cf. *amabilis* (Amblyoponinae) had one disulfide bond. This suggests that our study, despite sampling a broad range of ant subfamilies, cannot reveal the full extent of ant peptidome diversity. The vast number of stinging ant species (*ca* 9000) may reveal other peptide classes, structures and pharmacological properties. This is because specific genera like *Anochetus* and *Prionopelta* may have evolved different venoms based on atypical structural motifs.

LC/MALDI analysis and 2D landscapes

The mass fingerprinting of crude venoms revealed up to a maximum of only 42 peptides, as seen with *Solenopsis saevissima* venom. However, this low peptide count is consistent with results obtained from a series of tarantula venoms where the examination of crude venoms by MALDI-TOFMS revealed a maximum of 50 to 70 peptides.[53–56] Further examination of the HPLC fractions later revealed the full complexity of these tarantula venoms.[8] Crude venom mass fingerprinting is therefore a suitable tool for preliminary investigation and for comparing venom profiles in a given taxonomic group. However, it cannot reveal the full extent of the venom peptidome, probably due to ion suppression effects where large numbers of peptides compete for proton capture during the MALDI process. Consequently, the most abundant peptides, or the ones with the highest proton affinity, are likely to be over-represented in the mass spectra, while peptides with low abundance or lesser proton affinity may not ionise, or

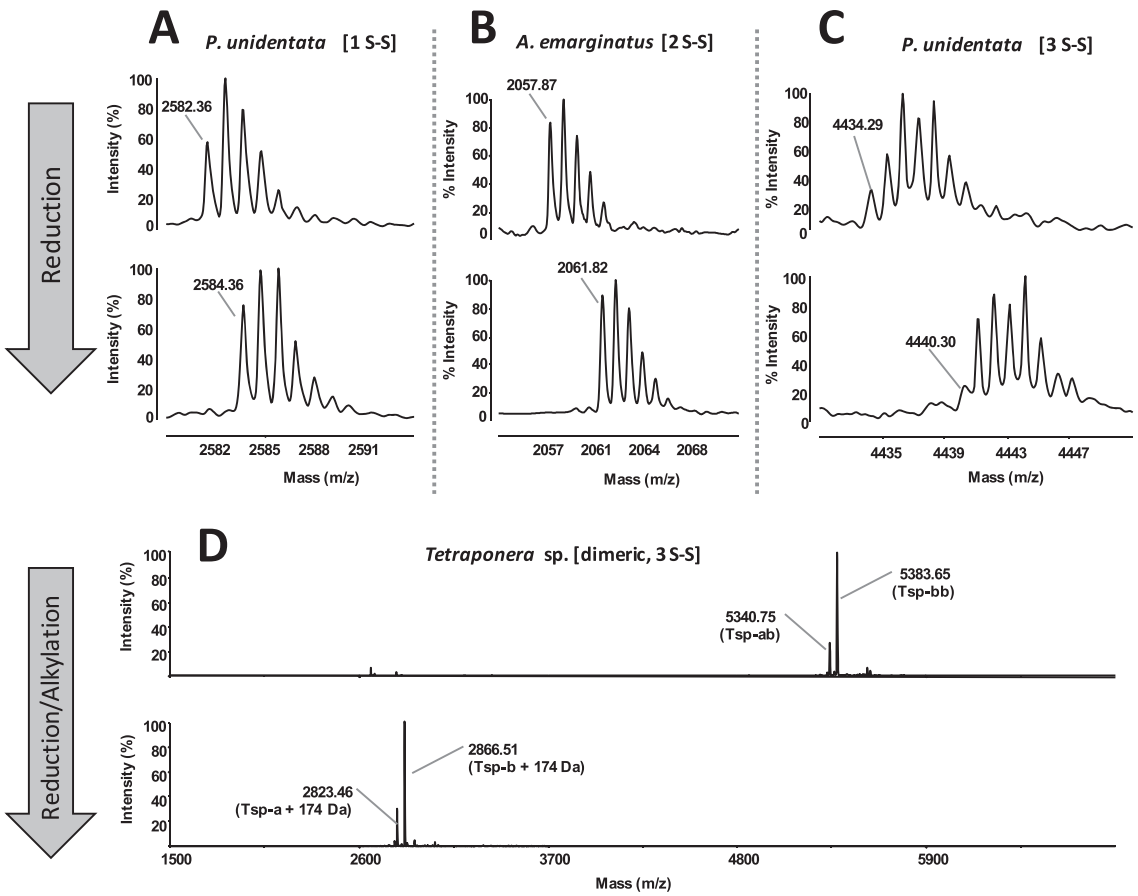

Figure 2. Identification of disulfide-bonded peptides in ant venoms. Typical MALDI-TOFMS spectra recorded in reflector mode showing $[M+H]^+$ ions of peptides increases of 2 Da (A; *Pachycondyla unidentata*), 4 Da (B; *Anochetus emarginatus*) and 6 Da (C; *Pachycondyla unidentata*) consistent with the presence of one, two and three disulfide bonds, respectively. Upper panels show native peptide isotopic clusters, while lower panels show the same peptide in reduced form obtained after reduction with DTT. (D) MALDI-TOF mass spectra from *f*16 of *Tetraponera* sp. venom before, and after, reduction and alkylation with IAA (R/A). Comparison of spectra before, and after, R/A revealed the presence of the heterodimeric peptide Tsp-ab (*m/z* 5340.75) and the homodimeric peptide Tsp-bb (*m/z* 5383.65). After R/A, $[M+H]^+$ ions of peptides Tsp-ab and Tsp-bb disappeared and the $[M+H]^+$ ion of the alkylated monomeric Tsp-a (*m/z* 2823.46) and Tsp-b (*m/z* 2866.51) appeared. This is consistent with *S*-carboxymethylation of three cysteines in each monomer.

ionise to provide signals below the detection limit. This results in spectra displaying only a subset of the crude venom complexity. The key to revealing the full extent of a venom peptidome diversity is chromatographic separation of the venom prior to mass analysis, using hyphenated chromatography either online (LC coupled to electrospray ionisation MS) or offline (LC coupled to sample deposition and MALDI-TOFMS). We therefore proceeded to fractionate a range of ant venoms into fractions containing fewer peptides to reveal the true venom complexity.

The resulting chromatographic profiles showed few similarities between the various subfamilies (Fig. 3) and varied in their complexity. This is reflected by the number of fractions collected from the chromatograms. A total of 27 fractions were collected from the least complex venom (*Tetraponera* sp.) and 61 from the most complex venom (*Eciton burchelli*). The overall complexity and range of elution patterns observed in ant venoms are parallel to those observed in the RP-HPLC profiles of other venomous arthropods such as Australian funnel-web spiders.[10] The eight ant venoms that were fractionated showed peptides eluting in a broad range of hydrophobicity. The most hydrophilic fraction eluted at 10.8% ACN (Fraction *f*1 from the venom of *Eciton burchelli* at 15.8 min) and the most hydrophobic fraction eluted at 60% ACN (*f*43 from the venom of *Pachycondyla goeldii* at 65 min). *Prionopelta* cf. *amabilis* venom eluted over a narrow hydrophobicity range (15.9–35.2% ACN) while both *Manica rubida* and *Pachycondyla goeldii* venoms eluted in a broad hydrophobicity range (13.1–57% ACN and 15–60% ACN, respectively). This highlights the broad physicochemical diversity among ant venom peptides, suggesting that peptides with different structural scaffolds or widely differing amino acid compositions are present.

The chromatographic fractions were then analysed by offline MALDI-TOFMS leading to the construction of bi-dimensional graphs, termed '2D venom landscapes', in which the molecular mass of each peptide in a given venom was plotted against the HPLC retention time reflecting hydrophobicity. The 2D landscapes revealed the enormous peptide complexity in

Rapid Communications in Mass Spectrometry

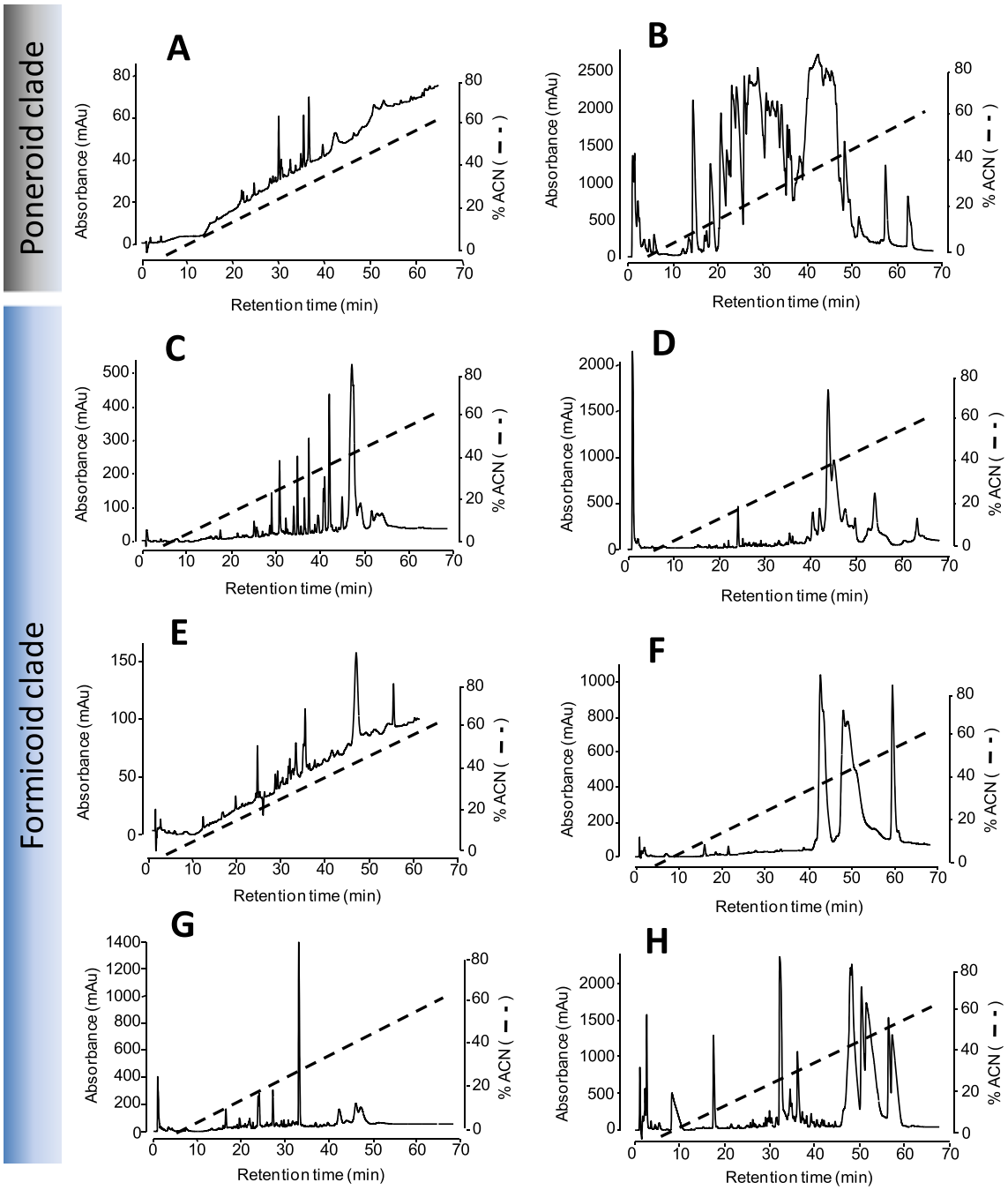

Figure 3. Representative RP-HPLC chromatograms of formicoid and poneroid ant venoms. Venoms were separated by C18 RP-HPLC using an ACN gradient of 1%/min (black dotted line). Panels show the chromatographic profile of venoms from (A) *Prionopelta* cf. *amabilis* (Amblyoponinae),(B) *Pachycondyla goeldii* (Ponerinae), (C) *Acanthostichus* sp. 1 (Cerapachyinae), (D) *Eciton burchelli* (Ecitoninae), (E) *Myrmecia pilosula* (Myrmeciinae), (F) *Tetraponera* sp. (Pseudomyrmecinae), (G) *Gnamptogenys sulcata* (Ectatomminae) and (H) *Manica rubida* (Myrmicinae).

ant venoms (Fig. 4). The total number of masses detected in the venoms analysed varied from 31 to 288 for *Prionopelta* cf. *amabilis* and *Pachycondyla goeldii*, respectively (Fig. 5). Some fractions were highly complex and, although they were collected as single peaks based on signal monitoring, mass analysis revealed the presence of up to 26 peptides in a single fraction (*f*18 at a retention time 32.6 min from *Pachycondyla goeldii*). This clearly demonstrates the power of the 2D analytical approach, as true venom complexity is not revealed by single-dimensional analysis.

Although many more peptides were detected, the mass distribution of peptides from the LC/MALDI-TOFMS analyses was quite similar to that previously observed in the analysis of the corresponding crude venoms. A total number of 1112 peptides were detected in the eight venoms by LC/MALDI-TOFMS with 94.4% of all peptides having a

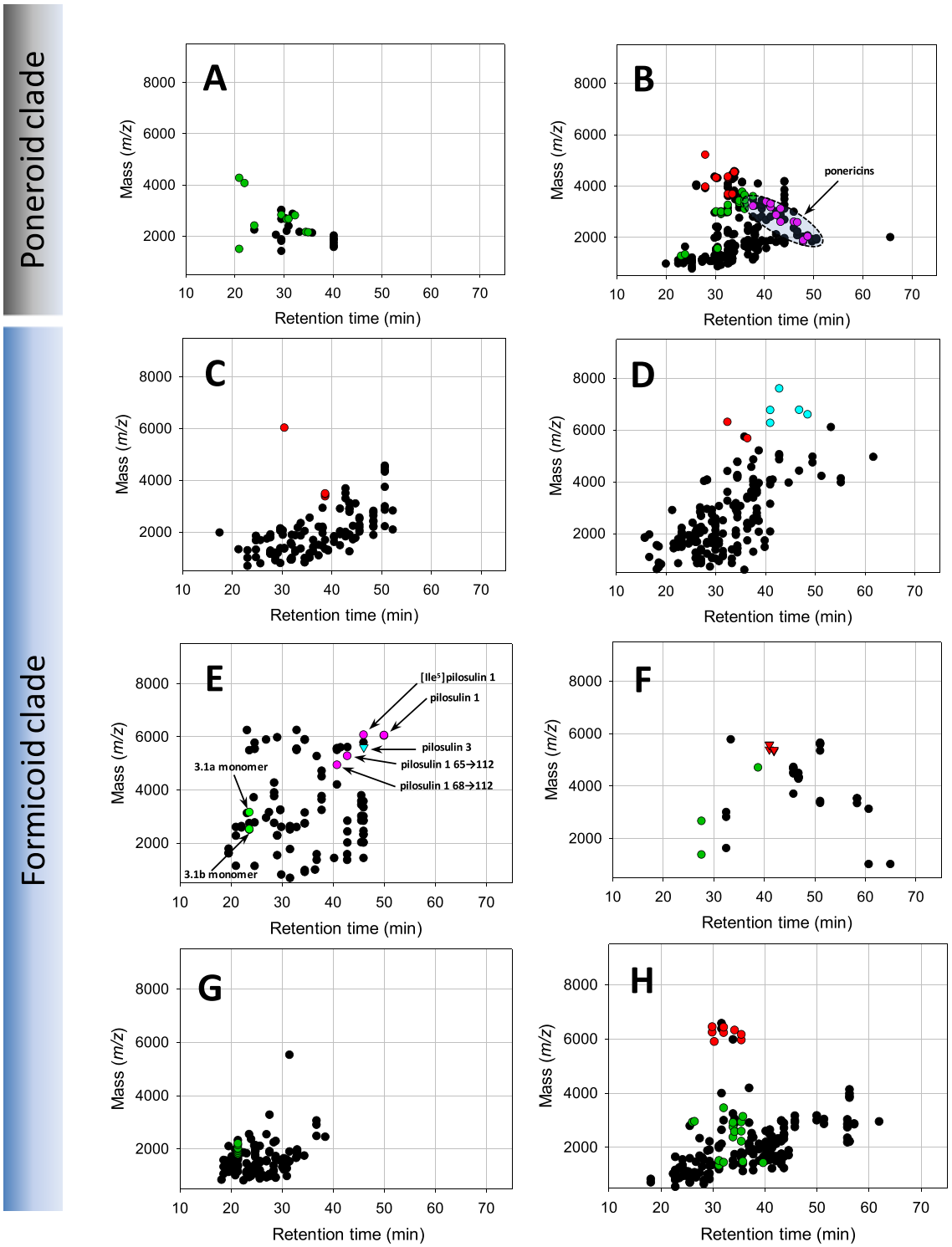

Figure 4. 2D landscapes of ant venoms from formicoid and poneroid ants. (A–H) Representative species from eight different subfamilies showing the LC/MALDI-TOFMS venom profiles of (A) *Prionopelta* cf. *amabilis* (Amblyoponinae), (B) *Pachycondyla goeldii* (Ponerinae), (C) *Acanthostichus* sp. 1 (Cerapachyinae), (D) *Eciton burchelli* (Ecitoninae), (E) *Myrmecia pilosula* (Myrmeciinae), (F) *Tetraponera* sp. (Pseudomyrmecinae), (G) *Gnamptogenys sulcata* (Ectatomminae) and (H) *Manica rubida* (Myrmicinae). Black circles indicate $[M+H]^+$ ions of peptides without disulfide bonds, green circles represent peptides with one disulfide bond, cyan circles $[M+H]^+$ ions of peptides with two disulfide bonds and red circles $[M+H]^+$ ions of peptides with three disulfide bonds. Red inversed triangles in the 2D landscape of *Tetraponera* sp. (F) show the presence of $[M+H]^+$ ions of dimeric peptides with three disulfide bonds while the cyan inversed triangle in the 2D landscape of *M. pilosula* (E) represents heterodimeric pilosulin 3 reticulated by two disulfide bonds. Purple circles highlight previously characterised pilosulins (E) and ponericins (B) found in *M. pilosula* and *P. goeldii* venoms, respectively.

Rapid Communications in Mass Spectrometry

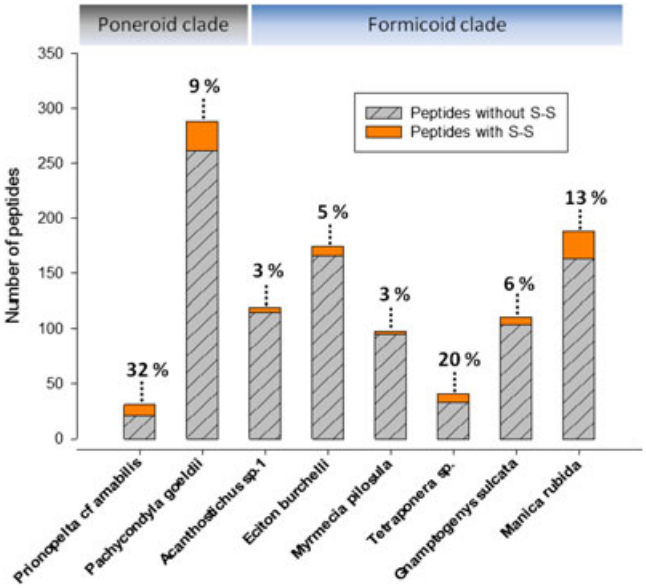

Figure 5. Total numbers of peptides in eight representative ant venoms revealed using LC/MALDI-TOFMS. Orange bars show the proportion of disulfide-bonded peptides detected by a mass shift after reduction of each RP-HPLC fraction with DTT. The percentages of disulfide-bonded peptides in whole venoms are indicated above each bar.

mass <5 kDa and 87.5% <4 kDa. However, most peptides in the venoms of formicoids yield $[M+H]^+$ ions that fall into a broad *m/z* range such as in *Eciton burchelli* (*m/z* 592.2–7595.0), *Myrmecia pilosula* (*m/z* 679.5–6246.4), *Tetraponera* sp. (*m/z* 1012.2–5773.2) and *Manica rubida* (*m/z* 535.4–6570.0), with many peptides having masses >4 kDa, while the other formicoid and poneroid venoms contain peptides that were almost exclusively lower than 4 kDa in mass (Fig. 4). In contrast, some venoms were mostly composed of peptides in a remarkably narrow mass range. This included venoms such as *Prionopelta* cf. *amabilis* that contained only 31 peptides with $[M+H]^+$ ions ranging from *m/z* 1411.1 to 4265.2 (Fig. 5).

Disulfide bond mapping

For a more precise characterisation of the disulfide-bonded peptides in each venom, the chemical reduction of each HPLC fraction was carried out to map the presence and number of disulfide bonds. We detected the presence of monomeric peptides with one, two or three disulfide bonds within all the venoms tested (Fig. 4). Peptides with one disulfide bond were found in a wide range of venoms including *Prionopelta* cf. *amabilis*, *Pachycondyla goeldii*, *Tetraponera* sp., *Myrmecia pilosula*, *Gnamptogenys sulcata* and *Manica rubida*. The *m/z* values of these $[M+H]^+$ ions ranged from 1274.2 (*Pachycondyla goeldii*) to 4701.8 (*Tetraponera* sp.). Peptides with two disulfide bonds were identified in the venom of *Eciton burchelli* within the range *m/z* 6594–7595 (Fig. 4(D)). Peptides with two disulfide bonds were also identified in the crude venom analysis of *Anochetus emarginatus*, *A.* cf. *diegensis* and *A. horridus* venoms. However, the *m/z* values of the $[M+H]^+$ ions of these peptides were much lower than those of *Eciton burchelli* (*m/z* 1623.6–2709.1), as shown in Supplementary Table S3 (Supporting Information). This difference in *m/z* ranges may indicate that these peptides belong to distinct toxin families. A group of 15 linear ponericins was previously characterized from the venom of *Pachycondyla goeldii*.[26] In the present study, 11 of these ponericins (i.e. G1, G2, G3, G4, G5, G6, L1, L2, W3 and W5) were also detected within the 2D venom landscape of *P. goeldii* (Fig. 4(B)).

The disulfide bond mapping of HPLC fractions revealed peptides with three disulfide bonds in the venoms of *Pachycondyla goeldii*, *Acanthostichus* sp. 1, *Eciton burchelli* and *Manica rubida* with $[M+H]^+$ ions within the broad range of *m/z* 3651.2–6440.8. Both homo- and heterodimeric peptides (Tsp-ab and Tsp-bb) were also found in the venom of *Tetraponera* sp. (Pseudomyrmecinae) with a mass shift, after reduction and alkylation, consistent with the presence of three disulfide bonds (Fig. 2(D)). It is presently unclear, however, if each Tsp monomer is linked to its other subunit by three inter-chain disulfide bonds or if each Tsp monomer has only one inter-chain disulfide and an additional intra-chain disulfide bond. Myrmexins are dimeric peptides that have been previously reported in the venoms of *Pseudomyrmex triplarinus* and *P. penetrator* (Pseudomyrmecinae).[44,57] However, myrmexin peptides only have two cysteine residues per monomer, not three. Although the linear pilosulin 1 (and its isoforms [Ile^5]pilosulin 1, pilosulin 1 65→112 and pilosulin 1 68→112) and the heterodimeric pilosulin 3 were found in the venom of *Myrmecia pilosula* (Fig. 4(E)), the homodimeric pilosulins 4 and 5 were not detected, although these peptides have been previously reported in *M. pilosula* venom.[42,43] Overall, the percentage of disulfide-linked peptides in venoms ranges from 3 to 20% for *M. pilosula* and *Tetraponera* sp., respectively (Fig. 5), highlighted by the dominance of linear peptides in ant venoms. However, the venom of *Prionopelta* cf. *amabilis* stood out with 32.3% of the 31 peptides having one disulfide bond (Fig. 5).

DISCUSSION

Ant venoms evolved bioactive peptides in order to disrupt multiple biological targets and permit the capture of arthropod prey, deter predators, communicate with other members of the colony and also act as antimicrobials. It is therefore not surprising that the present study of the ant venom peptidome has revealed enormous variation in the complexity of ant venoms highlighting the potentially diverse pharmacologies and functions of ant venom peptides. It is now well established from other taxonomic groups[7,8] that venoms are highly complex, containing up to 1000 peptides, and that many peptide classes as well as numerous isoforms in each class combine into a complex biochemical cocktail. Our investigation of ant venoms reveals that a wide range of ants produce venoms with a peptidic composition as complex as those of spiders, scorpions or cone snails as previously demonstrated in the venom of the ponerine ant *Dinoponera quadriceps*.[28] The LC/MALDI-TOF mass fingerprinting of a wide range of poneroid and formicoid ant venoms detected total numbers of peptides ranging from 31 to 288. Indeed, the venoms of *Pachycondyla* (Ponerinae) ants seem to be particularly rich in peptide components and the total numbers of peptides appear to be as great as in the previously described venom from the related ponerine ant, *Dinoponera quadriceps*.[28] Based on the present study, we would estimate that ant venoms contain on average 130

unique peptides. If we assume a total of *ca* 9000 stinging ant species, we can calculate the total number to be more than 1 million peptides in all stinging ant venoms. However, this number is probably an under-estimate, as this does not take into account the intra-species variations observed in ant venoms.[28] Moreover, cryptic ant species also contribute to a hidden peptide diversity as the venom peptide profile can vary among these species.[21] Indeed, many ant species are still undiscovered particularly within tropical areas and the total number of ants could reach 25 000 species (currently *ca* 13 000 species described).[58]

Hymenopteran venom peptides have not been extensively investigated to date. However, past studies on wasp venoms revealed small linear peptides[14,59–61] and some rare single disulfide-linked peptides such as pallipine-I, −II and -III from the venom of the wasp *Agelaia p. pallipes*[14] and sylverin from the venom of *Protonectarina sylveirae*.[62] A wide survey of ant venom peptidomes has never been attempted before and this study therefore constitutes the most extensive overview conducted to date, providing a broad overview of the variability of ant venom peptide composition. Ant venom peptides appear to be similar in mass to those of wasps as the majority of their peptides was also less than 4 kDa in mass and devoid of disulfide bonds. We have also shown that formicoid ants still possess some large peptides, consistent with earlier studies on ant venoms that showed peptides larger than 4 kDa among the formicoid subfamilies Ectatomminae, Pseudomyrmecinae and Myrmeciinae.[42–45,63] Given their impressive ecological diversity, formicoid ants may therefore have enhanced their venom arsenal by developing more complex and larger peptides that permit them to modulate novel pharmacological targets.

Disulfide-bonded peptides often act on ion channels and receptor targets and are common and dominant components in the venoms of a wide range of evolutionarily unrelated predators including sea anemones, cone snails, centipedes, scorpions, spiders and snakes.[11,33] The present study has highlighted the distribution of monomeric peptides structured by one, two or three disulfide bonds in a wide range of ant venom peptidomes. Based on the present study, such peptides appear to be minor components within most ant venom peptidomes except for venoms such as *Anochetus emarginatus*, *A.* cf. *diegensis*, *A. horridus* and *Prionopelta* cf. *amabilis* that are mostly composed of disulfide-linked peptides. Peptides with one disulfide bond have only been previously identified in the venoms of *Pseudomyrmex gracilis* and *P. penetrator*.[57] In the present study, peptides containing a single disulfide bond were extended to include venoms from five additional ant subfamilies encompassing 14 ant species (Supplementary Table S3, Supporting Information). In hymenopteran venoms, monomeric peptides reticulated by two disulfide bonds, such as apamin, were only described from the defensive venom of the honey bee *Apis mellifera*.[64] Importantly, the presence of 17 such peptides from three species of *Anochetus* is the first report of peptides in ant venoms with two disulfide bonds.

The *Dinoponera* ICK-like peptide[41] is the only monomeric peptide with more than one disulfide bond previously described from ant venoms. Peptides sharing high homology with an ICK peptide from tarantula venom have been found in the venom of the myrmecine ant, *Strumigenys kumadori*;[40] however, this data remains unpublished. Importantly, we found one peptide in the crude venom of *Pachycondyla unidentata* and several peptides from the venom of *P. goeldii* reticulated by three disulfide bonds that had masses within the most common peptide mass range of other ICK toxins (3500–4500 Da). Therefore, this strongly suggests that this toxin scaffold, which has evolved many times in unrelated venomous animals,[65] may also be present in many other ant venoms. However, the definitive identification of an ICK structural motif will require the purification of the peptides and determination of the disulfide linkage and tertiary structure. All the remaining disulfide-bonded peptides identified in ant venoms were homo- or heterodimeric complexes formed from monomers linked by one or two disulfide bonds from *Pseudomyrmex* spp. (Pseudomyrmecinae) and *Ectatomma* spp. (Ectatomminae) or *Myrmecia pilosula* (Myrmeciinae).[23,44,45,57,66] We also found dimeric peptides within the venom of the related pseudomyrmecinae ant, *Tetraponera* sp.

Except for the heterodimeric peptide pilosulin 3 ($[M+H]^+$ ion of *m/z* 5603), no other dimeric peptides were identified in either *Myrmecia pilosula*, *Ectatomma tuberculatum* or *E. brunneum* venoms, although several different dimeric pilosulins (*m/z* 8192–8540) and ectatomins (*m/z* 7923–9419) peptides have been fully characterised from these venoms.[42,43] The ability of our MALDI-TOFMS method using a FA/serine matrix to mainly detect $[M+H]^+$ ions in the *m/z* range *ca* 500–8000 may explain why the dimeric ectatomins and pilosulins were not detected in our investigation. This shows that the peptide richness is likely to be even more extensive. The use of complementary analyses, employing other techniques such as ESI-MS or using different matrices, may reveal the presence of larger peptides.

CONCLUSIONS

The present work constitutes the most extensive study of ant venom peptidomes and demonstrates the diversity in mass and disulfide connectivity of peptides in ant subfamilies and species. Ant venoms remain little investigated and the impressive diversity of peptides from ant venoms highlighted in this work combined with the improvement of mass spectrometry technology should boost future studies on ant venom toxins. Furthermore, the small sizes of ant venom peptides make the sequencing and synthesis of these toxins relatively simple therefore facilitating the characterisation of their pharmacological targets. Ant venoms may therefore provide a novel source of bioactive peptides to develop drug and bioinsecticide lead compounds.

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Rapid Communications in Mass Spectrometry

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